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Research Overview

Some Practical Considerations and Applications of the National Cancer Institute In Vitro Anticancer Drug Discovery Screen

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ABSTRACT During 1985-1990 the U.S. National Cancer Institute (NCI) phased out its murine leukemia P388 anticancer drug screening program and developed as the replacement a new in vitro primary screen based upon a diverse panel of human tumor cell lines. For each substance tested, the screen generates a remarkably reproducible and characteristic profile of differential in vitro cellular sensitivity, or lack thereof, across the 60 different cell lines comprising the panel. Several investigational approaches to display, analysis and interpretation of such profiles and databases, derived from the testing of tens of thousands of substances during the past 4-5 years since the NCI screen became fully operational, have been explored. A variety of useful, practical applications of the in vitro screen have become apparent. As these applications continue to evolve, they are proving to be complementary to diverse other anticancer screening and drug discovery strategies being developed or pursued elsewhere. Reviewed herein are some practical considerations and selected specific examples, particularly illustrating research applications of the NCI screen that may be more broadly applicable to the search for new anticancer drug development leads with novel profiles of antitumor activity and/or mechanisms of action.

Key Words: antineoplastics, cancer, drug development

INTRODUCTION

In simplest terms, the NCI in vitro primary screen consists of a panel of 60 different human tumor cell lines against which compounds are tested over a defined range of concentrations to determine the relative degree of growth inhibition or cytotoxicity against each cell line. The design and operation of the screen is such that, for each compound tested, both the absolute and relative sensitivities of individual cell lines comprising the screen are sufficiently reproducible that a characteristic profile or "fingerprint" of cellular response is generated. Depending upon the extent of

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differential cellular response, the profile may contain much information which is useful for further research.

The least interesting or useful (and expectedly most common) response to a random selection of chemical structures is none at all; that is, none of the cell lines show any evidence of growth inhibition or cytotoxicity. A similarly featureless profile may be obtained when one or more concentrations of the tested compound produce(s) growth inhibition and/or cytotoxicity of essentially the same magnitude across the entire panel of cell lines. Certainly, the NCI screen is capable of identifying highly potent, indiscriminate direct cell poisons, however that is not a unique or particularly useful attribute of the screen.

In contrast, the cell lines comprising the NCI panel may show differential sensitivity to a given test substance. The degree of differential response between the most and least sensitive lines typically may be relatively small (e.g., 2- to 10-fold), or occasionally as great as 3-4 orders of magnitude. Furthermore, the cell lines may be widely heterogeneous in response to a given compound, or they may be comparatively homogeneous, with only a relatively few lines showing much greater or lesser sensitivity than average. Regardless of the magnitude of the differential or the degree of heterogeneity of response of the cell line panel, it is the reproducibility of the response fingerprint that is key to the useful information contained therein. This valuable information can be exploited productively in its own right, as well as in complement to other drug discovery research models and strategies.

Routine operation of the NCI in vitro screen began in 1990, after five years of extensive development and pilot evaluations during 1985-89. Reviews of the concept, rationale and technical aspects of development of the screen are available elsewhere (e.g., see Boyd, 1986,1989,1993; Boyd et al., 1992). From 1990 to the present, more than 30,000 compounds, submitted by cancer researchers worldwide, have been tested in the NCI screen. Screening databases derived therefrom have provided NCI staff and collaborators a unique opportunity to explore a considerable variety of data analysis strategies and methods. Reviews and other publications describing such studies are available (e.g., see Paull, et al. 1989; Boyd, et al. 1992; Hodes, et al. 1992; Weinstein, et al., 1992; Weinstein, et al. 1994; van Osdol, et al. 1994; Paull, et al. 1994).

In many if not most of the important respects thus far examined, the results and conclusions from diverse analytical approaches have been convergent. Increasingly sophisticated mathematical and computational techniques are being developed and applied further, and undoubtedly these will add important new dimensions to the valuable information that can be derived from the in vitro screening panel. This seems particularly certain when the data from parallel ongoing efforts to further characterize the unique biology of the individual cell lines can be further integrated into the analyses

The purpose of this brief review is to offer some practical considerations and to describe and illustrate some relatively simple and straightforward research applications that may be of immediate and considerable utility to many current and future users of the NCI service screen. In so doing, some selected examples are drawn from the authors' particular research experiences using the screen. The review is by no means intended to be comprehensive; the scope is limited to some generally useful applications that can be pursued by "nonexperts" using relatively simple analytical techniques with data generated and supplied routinely for pure compounds submitted for testing in the NCI screen.

THE SCREEN

Detailed descriptions of the screening assay in use as of 1990 are available elsewhere (Boyd,1989; Monks et al.,1991; Skehan et al., 1990). Some changes in the screen subsequently have been made, particularly in late 1992. These are noted briefly below. Investigators evaluating recent data in comparison with older data from the NCI in vitro screen may wish to take these differences into account.

Cell Line Panel

The identities, sources, derivation, morphological and immunocytochemical characteristics, and methods of maintenance of the cell lines comprising the NCI 60 cell line panel as of 1990, have been described in detail elsewhere (Boyd, 1989; Monks et al., 1991; Stinson et al., 1992). In December 1, 1992, ten of the original cell lines were removed from the panel to make way for ten breast cancer and prostate cancer lines. The lines removed from the panel comprised: [lung] HOP-18, LXF-529L, DMS114, DMS273; [brain] XF-498, SNB-78; [colon] KM20-L2, DLD-1; [renal] RXF-631 L; [melanoma] M19MEL. The lines added (and references to the original sources and/or corresponding descriptive publications) are as follows: [breast] MCF-7 (Soule et al. 1973), MCF-7ADR (Cohen et al., 1986), HS578T (Hackett et al., 1977), MDA-MB-231 (Cailleau et al.,1974; Siciliano et al.,1979), MDA-MB-435 (Cailleau et al., 1978; Brinkley et al., 1980), MDA-N (P. Steeg, NCI, unpublished), BT-549 (ATCC, Rockville, MD, unpublished), T47-D (Keydar et al.,1979); [prostate] DU-145 (Stone et al., 1978; Mickey et al., 1977), PC-3

(Ohnuki et al., 1980; Kaighn et al., 1979; Kaighn et al., 1981).

Screening Assay

In routine screening, each agent is tested over a broad concentration range against every cell line in the panel. All lines are inoculated onto a series of standard 96-well microtitre plates on day zero, followed by a 24 hr incubation in the absence of the test compound. The inoculation densities employed depend upon the particular cell line and its growth characteristics. Inoculation densities used currently in the screen for many of the cell lines are the same as originally published (Monks et al., 1991). Exceptions, introduced as of December 1, 1992, are as follows (current densities used [cells/well] are indicated in parentheses): HOP-62 (10,000), UO-31 (15,000), 786-0 (5,000), LOX IMVI (7,500), SR (20,000). Inoculation densities used for the breast and prostate lines beginning with their addition on December 1,1992 are as follows: MCF-7 (5,000), MCF-7ADR (15,000), HS578T (20,000), MDA-MB-231 (20,000), MDA-MB-435 (15,000), MDA-N (15,000), BT-549 (20,000), T47-D (20,000), DU-145 (15,000), PC-3 (7,500). Further exceptions introduced as of July 25,1994, are: NCI-H226 (15,000), RXF393 (15,000), ACHN (10,000), PC-3 (7,500). Test compounds are routinely evaluated at five, 10-fold dilutions starting from a high of 10^{-4} M, unless otherwise requested. Following a 48-hour incubation with the test compound, the cells are assayed by the sulforhodamine B procedure (Skehan et al.,1990; Monks et al., 1991; Rubinstein et al., 1990). Optical densities are measured on automated plate readers, followed by acquisition, processing, storage and availability for display and analysis on a microcomputer.

DATA DISPLAY AND ANALYSIS

A detailed description of the contents and format of the data report package routinely provided to submitters of compounds for NCI screening has been published (Boyd et al., 1992). The "dose-response matrix" part of the package is no longer provided or routinely used. The "dose-response curves" and the "mean-graphs" components of the report are the main interest of most investigators. Therefore, following are some brief descriptions and comments concerning the dose-response curves, calculated response parameters and mean-graphs which are most germane to the examples to be presented. Also offered are some comments and suggestions as to standards for investigator reporting of NCI screening data in the scientific literature. The consistency, detail, format and placement of such information has increasingly been a concern of some journal editors (e.g., see Editors, 1994).

Dose-Response Curves

Each successful test of a compound in the full screen generates 60 dose response curves, which are printed in the NCI screening data report as a series of composites comprising the tumor-type subpanels, plus a composite comprising the entire panel. Data for any individual cell line(s) failing quality control criteria, or otherwise deficient for any cell line(s) not tested successfully, are eliminated from further analysis and are deleted from the screening report. Figure 1 shows contrasting patterns in the dose-response curves obtained from two different compounds. The figure was prepared directly from the corresponding NCI supplier reports by deleting extraneous or otherwise distracting information, and adding minimal scaling and reference information for clarity and substantial photoreduction. The cell line subpanels are identified in the figure legend (Fig.1).

The "percentage growth" (PG) term, and meaning of the +50, 0 and -50 response reference lines, and the calculated response parameters, GI_{50}, TGI, and LC_{50} have been defined elsewhere (see Boyd et al., 1992; Monks et al., 1991). Although the response parameters are already calculated by computer and provided to the investigator in the data report package, it is important to appreciate how these values are determined, and likewise how this may affect data interpretation.

The 50% growth inhibition parameter (GI_{50}) is the concentration of test drug where 100 x (T-To) / (C-To) = 50 = PG. The optical density of the test well after the 48 hour drug exposure is T; the optical density at time zero is To; and, the control optical density is C. The PG is a T / C- like parameter that can have values from +100 to -100. Whereas the GI_{50} may be viewed as a growth-inhibitory level of effect, the TGI signifies a "total growth inhibition" or cytostatic level of effect. The TGI is the drug concentration where 100 x $(T-T_0)$/ (C-T) = 0 = PG. The LC_{50} is the lethal concentration, "net cell killing" or cytotoxicity parameter. It is the concentration where 100 x $(T-T_0)$ / To = -50 = PG. The control optical density is not used in the calculation of LC_{50} .

The GI_{50}, TGI and LC_{50} values are calculated by interpolation using the tested concentrations that give PG values above and below the respective reference values (e.g., 50 for GI_{50}). Therefore, a "real" value for any of the three response parameters is obtained only if at least one of the tested drug

concentrations falls above, and likewise at least one falls below, the respective appropriate PG reference value (i.e., in other words, the dose-response curve for that particular cell line must cross the respective PG reference line). If, however, for a given cell line all of the tested concentrations produce PG's exceeding the respective reference level of effect (PG value of +50, 0 or -50 as appropriate), then the lowest tested concentration (specified in negative $\log_{10}$ units) is assigned as the default value. In the screening data report, that default value is preceded by a "<" sign, signifying that the "real" value is something "less-than" the lowest tested concentration. Likewise, if none of the tested concentrations produces the required PG reference level of effect or greater, then a ">" sign precedes the printed default value (which is the highest tested concentration or HICONC, specified in negative $\log_{10}$ units), signifying that the "real" value is something "greater-than" the HICONC. In any case, either the "real" (interpolated) or the default (< or >) GI_{50}, TGI and LC_{50} for every cell line in the panel are printed with the mean-graphs included in the screening data report. The investigator can, if desired, verify visually that for any printed response parameter concentration preceded by a "<" or a ">" for a given cell line in the GI_{50}, TGI or LC_{50} mean graphs, there must be a corresponding dose-response curve that either lies entirely *below* or entirely *above* the corresponding PG reference line, respectively.

For some applications, the occurrence of many default values for the response parameters in a given screening test can have a major impact on both the accuracy and the interpretation, and therefore the usefulness of the data. This problem may be particularly prominent, for example, in structure-activity studies where both quantitative (e.g., overall or panel-averaged potency) and qualitative (e.g., profile of differential cytotoxicity) comparisons of compounds are desired. For any given compound, the particular range of concentrations tested can be the major determinant of the extent of occurrence of "<" or ">" response parameter values. Therefore, it may be necessary to obtain further testing of a compound in concentration range(s) other than employed routinely in the screen, depending upon the intended use of the data. Indeed, in certain instances, data from the testing of a given compound in different concentration ranges may yield distinctly useful, non-overlapping information. Examples that follow may provide further clarification of these points. Before presentation of specific examples, however, some additional background and descriptive information concerning the "mean-graph" and the COMPARE analysis concepts are pertinent.

Mean-Graph

A mean-graph is a pattern created by plotting positive and negative values, termed "deltas", generated from a set of GI_{50}, TGI, or LC_{50} concentrations obtained for a given compound tested against each cell line in the NCI in vitro screen. Figure 2 shows the GI_{50}, TGI and LC_{50} mean-graphs derived from the dose-response data of Figure 1. This figure was also prepared directly from the NCI supplier report, by manually cropping and editing the original mean graphs.

The deltas are generated from the GI_{50}, TGI or LC_{50} data by a three-step calculation. For example, the GI_{50} value for each cell line successfully tested against a given compound is converted to its $\log_{10}$ GI_{50} value. The mean panel $\log_{10}$ GI_{50} value is obtained by averaging the individual $\log_{10}$ GI_{50} values. Both "real" and default values are used in the calculation. Each individual $\log_{10}$ GI_{50} value then is subtracted from the panel mean to create the corresponding delta.

To construct the mean-graph, the deltas are plotted horizontally in reference to a vertical line that represents the calculated mean panel GI_{50}. The mean panel GI_{50} may or may not represent, nor even approximate, a "true" mean, depending upon the extent to which defaults were among the values averaged (see previous section). In any case, the negative deltas are plotted to the right of the mean reference line, thereby proportionately representing cell lines more sensitive than the calculated average. Conversely, the positive deltas are plotted to the left of the reference line to represent the less sensitive cell lines to the given agent. Thus, for example, a bar projecting 3 units to the right of the vertical reference line in a GI_{50} mean-graph indicates that the GI_{50} concentration for that cell line is 1000 times less than the panel- averaged GI_{50} concentration. The TGI and LC_{50} mean-graphs are prepared and interpreted similarly.

In the full standard NCI screening data report package, three additional numbers are printed at the base of each of the three respective mean-graphs provided. These numbers are the MG-MID, the Delta (not be confused with the "delta" for an individual cell line), and the Range. The MG-MID is the calculated mean panel GI_{50}, TGI or LC_{50}. The Delta is the number of $\log_{10}$ units by which the delta of the most sensitive line(s) of the panel differs from the corresponding MG-MID. Similarly, the Range is the number of $\log_{10}$ units by which the delta of the most sensitive line(s) of the panel differs from the delta of the least sensitive line(s). The MG-MID, Delta and Range are most meaningful when few if any default values are contained in the corresponding mean graph; otherwise

they are not particularly meaningful or useful, and indeed can be misleading. Further clarification of this point follows in a discussion of data presented in Figures 3, 4, 5A and 5B.

COMPARE

COMPARE is a computerized, pattern-recognition algorithm which has considerable utility in the evaluation and exploitation of data generated by the NCI screen. In essence, COMPARE is a method of determining and expressing the degree of similarity, or lack thereof, of mean-graph profiles generated on the same or different compounds. An early impetus for the creation of such a tool during the developmental phase of the screen was the need to standardize the screen and to establish and monitor its consistency and reproducibility over time. This was, and still is, accomplished by the regular testing of standard compounds which are expected to generate the same or very similar profiles when screened repetitively against the same panel of cell lines.

Further in the course of standardizing the screen, we selected as reference compounds approximately 170 agents for which a considerable amount of information was available about their preclinical and/or clinical antitumor properties and presumed mechanism(s) of action. These compounds included commercially marketed anticancer drugs, investigational anticancer drugs, and other candidate antitumor drugs which were or had been in preclinical development at NCI based upon activities in other cancer-related test systems. The repetitive periodic screening of these prototype "standard agents" has continued to the present, and remains the basis for calibration and standardization of the screen.

The standard agent database also is the key to many useful research applications of the NCI screen. For example, the response profile fingerprint of a selected standard agent may be used as the "seed" to probe any other available mean-graph database to see if there are any closely matching profiles contained therein. Conversely, a profile selected from any available mean-graph database can be used to probe the standard agent database to determine if there are any closely matching standard agent profiles. Databases used for such studies may be constructed or defined as desired and may be relatively small (e.g., a selected congeneric series of compounds) or very large (e.g., the entire database from all pure compounds tested in the NCI screen to date).

Initial NCI studies with COMPARE led quickly to the observation that compounds matched by their mean-graph patterns often had related chemical structures. Closer examination of this phenomenon led further to the realization that compounds of either related or unrelated structures and matched by mean-graph patterns frequently shared the same or related biochemical mechanisms of action (e.g., see Boyd, 1993, and Paull et al., 1994, and references therein). Before preceding to more specific examples illustrating some of the intriguing research applications of this phenomenon, further description of the COMPARE calculation methodology is in order.

Method of COMPARE Calculations

COMPARE analyses may be performed using the mean-graph deltas calculated from either the GI_{50}s, the TGIs or the LC_{50}s. When a selected particular mean-graph profile or "seed" is used to probe a given database, the appropriate delta value for each cell line is compared to the corresponding delta value for the same cell line for every mean-graph entry in the specified database set. If either delta value is missing for any cell line (e.g., due to test failure or quality control deletion), then that cell line is eliminated entirely from the calculation for that particular seed/mean-graph and database/mean-graph pair. Thus, for each mean-graph in the specified database a set of pairs (maximum of 60) of delta values is obtained. The commercially available SAS statistical program is used to calculate a Pearson product moment correlation coefficient (0.0-1.0) for each set of delta value pairs. Then the mean-graphs of all compounds in the specified database can be rank-ordered for similarity to the seed mean-graph.

Impact of Default Values for Response Parameter Concentrations

Default GI_{50}, TGI or LC_{50} values (see defined above) are included in the mean-graph and COMPARE because they represent valued information even though the information is less exact than the measured values would be if the measured values were available. However, this can sometimes lead to peculiarities, of which the investigator should be aware. For example, in an extreme case where a compound has essentially no effect at the highest tested concentration, and therefore the GI_{50}s, TGIs and LC_{50}s are all represented by the HICONC default, the corresponding mean-graphs appear as a flat vertical line, and COMPARE has no meaningful pattern to correlate. In an opposite extreme case, the tested compound is sufficiently potent that the lowest concentration tested is the default value for all of the GI_{50}s, TGIs and LC_{50}s. In this instance, the corresponding mean-graphs are also flat vertical lines, and COMPARE has nothing to meaningfully correlate. Between such extremes are examples of mean-graphs

with few, many or no default-value GI_{50}s, TGIs or LC_{50}s.

Mean-graphs containing many default values can give good results in COMPARE, but the possible presence of the default values requires an additional strategy in the database preparation. This is necessary because the extent of default values in the mean-graph of a particular compound depends upon the particular range of tested concentrations for that compound. For a given compound, the database may contain multiple mean-graphs from tests at different concentration ranges. Moreover, the database may contain multiple mean-graphs from replicate testing of the compound in a given concentration range. The investigator may wish to obtain an "averaged" mean graph for publication or further analysis, or for use as a seed in a COMPARE study. Therefore, the strategy we use is to group the data for a given compound according to the HICONC used in the individual screening experiments. Thus, if multiple tests of a compound are present in the database, only those experiments with the same HICONC are averaged. Since there may be differences in the default-value content of the averaged data, and because the averages may be calculated from different experiments, the COMPARE-generated correlation coefficients may be different for the same compound tested at different HICONCs. Moreover, the COMPARE user has the option to choose any one of the HICONC sets for the probe pattern averaging, or the user may choose to average all seed data regardless of the HICONC.

The investigator should also be aware that when "averaged" mean-graphs are printed, there are no "~" or ">" signs shown for any of the individual averaged GI_{50}, TGI or LC_{50} values for each cell line, even though their calculation may have included one or more default values. In the extreme case for a given cell line, the averaged value shown for the GI_{50}, TGI or LC_{50} may in fact be equal to the single-test default value; this would be the case when all of the individual test values used to make the average were defaults. The extent to which default values comprise the averaged mean-graph derived from multiple tests of a given compound can best be ascertained by the investigator by an actual examination of the corresponding screening data from each of the individual tests used to make the average.

For routine COMPARE analyses the above considerations generally may be much less important than when the investigator wishes to determine an accurate and meaningful panel-averaged GI_{50}, TGI, or LC_{50} value. Such values may provide the best means to compare the relative overall potencies of related or unrelated compounds against the full cell line panel. For screening data from either a single test or multiple (averaged) tests, the extent to which default values comprise the individual or composite data used to calculate the overall panel-averaged GI_{50}, TGI or LC_{50} value, will determine the true accuracy and usefulness of the respective mean response parameter as a measure of comparative potency. Figures 3 and 4 illustrate two mean-graphs which differ markedly in their content of default values. The dose-response curves from which the mean-graphs of Figures 3 and 4 were derived are shown in Figure 5A and B, respectively.

Certain compounds tested in the NCI screen give peculiar "dose-response" data such as exemplified in Figure 5A. Indeed, there may be no apparent concentration dependency of the growth-inhibitory or cytotoxic effects of the given compound within the tested range of concentrations. Instead, the dose-response "curves" are actually just flat lines clustering above and below the TGI reference line; typically, very few if any of the dose-response lines cross any of the response parameter reference lines. Therefore, the corresponding derived GI_{50}, TGI and LC_{50} mean graphs are comprised almost entirely of default values, as exemplified by the TGI mean graph in Figure 3. The major impact upon the visual appearance of the mean-graph is vividly shown; at first glance there appears to be an extreme range of differential sensitivity of the cell lines to the given compound. However, this is artifactual; the computer has simply plotted the default-derived deltas corresponding to the highest or lowest test concentrations. Likewise, on the same basis the values given for the Delta and Range are artifactual. Similarly, the MG-MID value does not reflect any meaningful panel-average sensitivity of the cell lines to the given compound; it is therefore not a useful value for comparing the overall potency of the given compound to any related or unrelated compound. These potential problems in interpretation for the given compound can be remedied by testing at lower concentrations.

Figure 5B shows the result of testing the same compound at a later date in a much lower concentration range. In this instance, almost all of the dose-response curves cross the GI_{50} reference line, and therefore yield "real" GI_{50} values for the corresponding mean graph. Indeed multiple tests of this compound gave "real" GI_{50} values for all of the cell lines, with only an occasional sporadic default value for one or only a very few cell lines. Therefore, in Figure 4 the given Delta and Range values quite accurately reflect a true range of differential sensitivity among the full panel of cell lines to this agent. Likewise, the given MG-MID value quite accurately reflects a true overall panel-average sensitivity of the cell lines to this agent, and therefore is

a useful basis for comparison of overall potency of the given agent with related or unrelated compounds.

Considerations for Structure-Activity Study Strategies

The NCI screen provides an unprecedented tool for structure-activity relationship (SAR) investigations, by providing for simultaneous comparisons of quantitative (e.g., relative potency) as well as qualitative (e.g., selectivity profile and/or presumptive mechanism of action) features among a selected group of compounds. Lead optimization strategies may therefore be pursued to improve overall potency, to retain or diverge from a particular selectivity profile or mechanism of action, or to achieve a combination of these goals. In so doing, it may be necessary to test each compound of interest in two or more different, but preferably overlapping, concentration ranges. Also, it is highly desirable to subject each compound for a given SAR study series to multiple (e.g., triplicate or quadruplicate) tests in order to obtain averaged response
parameter values for more robust and statistically defensible analyses and comparisons.

It should also be noted that for a given compound, the tests obtained in one concentration range may be better for a given type of analysis or comparison, whereas the tests obtained in a different concentration range may be better for another type of analysis or comparison, and vice versa. For example, paradoxically, COMPARE analyses (e.g., qualitative comparisons) using the data in Figure 3 as the seed against the profiles of structurally and/or mechanistically related compounds yielded higher correlation coefficients than when similarly using the data of Figure 4 for the same compound. On the other hand, as already suggested above, the most meaningful quantitative (e.g., overall potency) comparisons for the same given compound were obtained with the data in Figure 4. It should be appreciated that the relative utility of the screening data for a given type of analysis for a given compound tested at a given concentration range may or may not parallel this particular example. The investigator should determine on a case-by-case basis which of the available or obtainable data are most appropriate, adequate or optimal for the desired analysis.

Finally, in reference to Figures 3 and 4 there is another precaution that should be noted. As can be seen from the listing of subpanel and cell line identifiers on the mean-graphs, the data in Figure 3 must be from the testing of the given compound prior to December 1, 1992, before the introduction of the breast and prostate cancer lines. Similarly, the data in Figure 4 must be derived from the testing of the same compound after December 1, 1992 (i.e., after the removal and addition of the various specific panel cell lines as described in a previous section). For any attempted quantitative or qualitative comparison or analysis of screening data for a given compound or compounds tested at different times, the investigator should take carefully into account when specific changes may have been introduced into the screening panel or assay protocol, relative to when the various data used in the analyses were generated. Without doubt, the best data for structure-activity investigations are those generated as nearly contemporaneously as possible for the entire set of compounds and standards of immediate interest.

EXAMPLES

Some further consequences of such analyses, options and strategies as identified above are discussed in further detail elsewhere (Paull et al., 1994). However, for purposes of the present review, we wish to emphasize the very simplicity of the concept and routine utility of COMPARE. Investigators may choose to perform their own calculations and analyses; indeed, they need not rely at all upon NCI databases, staff or computers to derive meaningful and useful COMPARE-type analyses from the NCI-supplied in vitro screening data on their compounds, and/or with like data on other compounds reported in the literature. However, in that respect, the adoption of consistent literature reporting standards and formats by users of the NCI screen is an important precedent. Some more specific examples follow to further illustrate these points.

Finding New Members of Known Mechanistic Classes

By regular application of COMPARE, using selected prototype seed compounds from the standard agent database, NCI staff maintain ongoing surveillance of the total historical screening database accrued from inception to date. In this manner, compounds with screening fingerprints matching standard agent(s) having known or presumed known mechanism(s) of actions can be identified and selected for further study. Follow-up (e.g., specific biochemical or molecular) studies can then be undertaken to confirm or disprove the identity or similarity of the mechanism of action of the selected compound(s) to that of the seed standard agent(s) upon which its selection was based. In this manner, NCI staff have been able to associate and subsequently confirm the database classification of compounds of previously unknown mechanisms of

action into a number of different known mechanistic classes of interest. For example, new members have been classified within general mechanistic categories of tubulin-interactive antimitotics, antimetabolites, alkylating agents, topoisomerase inhibitors, DNA binders, and the like. These and numerous other examples resulting from this kind of database prospecting have been published elsewhere (e.g., see Paull et al., 1992 and 1994, and references contained therein). However, in practical terms such studies are principally the purview of NCI staff having access to the full historical databases.

The more general user of the NCI screen can perform an analogous exercise using the screening data in hand on a compound of interest to obtain at least a preliminary indication of relatedness or dissimilarity to any known mechanistic class. In this case, the drill is to compare a profile of interest to the readily accessible standard agent database or to some selected individual member(s) thereof. Likewise, a profile of interest can be compared to other profiles which are suitably documented in the published literature, irrespective of whether any of the profiles to be compared are from compounds of known or unknown mechanistic classes. Some recent examples are especially pertinent to these points.

Pettit et al. [1993a, b, c, d] have described a series of unprecedented macrocyclic lactone polyethers named spongistatins (e.g., see Figure 6). These exceedingly potent cytotoxins were isolated during the course of in vitro murine P388 leukemia cytotoxicity bioassay-guided fractionation of extracts of certain marine sponges. To obtain a preliminary indication of the relative potency against the NCI panel of human tumor lines, and to ascertain preliminarily any possible relatedness to any known mechanistic class, the first available sample of the lead compound, spongistatin-1 (Figure 6) was tested in the NCI screen, then analyzed by COMPARE against the standard agents. Figures 3 and 5A are the actual screening results from that test. Clearly the compound was highly potent, and it produced peculiar dose-response and mean-graph profiles (5A), which experienced users of the screen will recognize are both typical and highly characteristic of the general mechanistic class comprising tubulin-interactive antimitotics. Indeed, COMPARE analyses confirmed a high correlation of the spongistatin-1 TGI mean graph profile (Figure 3) with the profiles of known antimitotic agents (e.g., vincristine, vinblastine, taxol, maytansine, rhizoxin, podophyllotoxin) which are known to act at the level of tubulin, albeit in more or less subtly different ways. Although the cell line screen cannot differentiate among different mechanistic subtypes within the general tubulin-interactive mechanistic class (for further discussion of this point, see Paull et al., 1992), it nonetheless can be exceedingly useful to focus the follow-up biochemical or other specific mechanistic studies of relevance. For example, subsequent investigations of spongistatin-1 as reported by Bai et al. (1993) both confirmed a tubulin-related mechanism of cytotoxicity and revealed distinctive differences from the various standard prototypes comprising the class.

Some months following the testing reflected in Figures 3 and 5A, additional amounts of spongistatin-1 were available for further testing, as were also sufficient amounts of spongistatins-2 through -7 (Figure 6). To enable an accurate comparison of both the relative overall potencies and patterns of differential cytotoxicities among the series, all seven compounds were tested contemporaneously, in quadruplicate, in each of several different overlapping concentration ranges. From the collective set of data thus generated, the most appropriate subsets were selected for the desired comparisons and analyses. Spongistatin-1 was used as the "benchmark" compound for the comparisons, and the most recent data for that compound were used for the analyses. Figures 4 and 5B actually are the data from one of the retests of spongistatin-1 done at a much lower concentration range than used for the original test Figures 3 and 5A.

Table 1 is a summary of the key results derived from the comparative study of the spongistatins. It was apparent that, as a group the spongistatins were highly potent, however there were significant individual differences in the average overall potency of the compounds to the panel of cell lines. On the other hand, the uniformly high COMPARE correlation coefficients indicated that all of the compounds of the tested series shared essentially the same mean-graph profile (and presumably therefore the same or very similar mechanism of action) as the selected benchmark compound, spongistatin-1. This is in contrast to the following examples which show that relatively subtle structural variations within a chemically related series of compounds may result in very great differences in differential cytotoxicity profiles (and therefore presumably differences in mechanism(s) of action) with or without any significant differences in overall panel-averaged potencies.

Finding New Mechanistic Classes and New Members Therein

Occasionally during the past several years of routine operation of the NCI in vitro screen, compounds have been detected which produce very striking differential cytotoxicity fingerprints which are highly

reproducible and characteristic, yet which bear little or no resemblance to the fingerprint of any standard agent, nor indeed to that of any known compound in the entire database of tested compounds. For instance, a pentahalogenated monoterpene, named halomon (Figure 7, structure 1), a natural product isolated from the red alga, Portieria hornemannii, produced one of the most extreme examples of differential cytotoxicity that had been seen in the NCI screen (Fuller et al., 1992). The dose-response curves of Figure 1A and the corresponding mean-graphs of Figure 2A are actually from the screening of halomon.

Some more recent studies (Fuller et al., 1994) of halomon and related halogenated monoterpenes also isolated from the same red alga have additionally revealed some interesting structure-activity requirements within the series, and have further established the novelty of the lead. Table 2 shows the results of comparative evaluations of these compounds in the NCI screen. Using the screening profile of halomon as the seed or benchmark for the comparisons, the profiles of compounds 2-4 were highly correlated to that of halomon itself. In contrast, the profiles of 5-8 appeared entirely unrelated, indeed essentially featureless and uninteresting. For example, the dose-response curves and corresponding mean-graphs of Figures 1 B and 2B, respectively, are actually from the screening of compound 7.

The mechanism(s) of in vitro cytotoxicity of halomon, and the differential cell line selectivity thereof, presently remain unknown. The striking contrasts even within the limited series 1-8 (Figure 7; Table 2) suggest that the lead and the similarly profiling compounds 2-4 are not acting merely as electrophiles (alkylating agents). Indeed, COMPARE analyses show no significant similarity of the halomon profile to that of any known alkylating agent, nor in fact to any known mechanistic prototype.

Another example of a novel profile of in vitro antitumor specificity revealed by the NCI screen is that of a series of quaternized ellipticines, which are preferentially cytotoxic to the brain tumor cell line subpanel (Acton et al., 1992; Acton et al., 1994). This is one of the first so- called "disease- oriented" (Boyd, 1989) or subpanel- specific leads detected by the NCI screen. Such leads have thus far proved quite rare among all compounds screened to date, but nonetheless are very intriguing.

The GI_{50}, TGI and LC_{50} mean-graph profiles of the acetate salt of the selected benchmark compound (Figure 8, structure 8) are shown in Figure 9. It should be noted also that Figure 9 is yet another example of a "scissors-and-tape" composite prepared from the original mean-graphs provided in the standard supplier report format. The figure is suitably simplified and stylized to allow reduction to a still-legible size typical of a 1-column width in many scientific journals. However, the routine publication of mean-graphs from NCI screening of compounds may not be appropriate or desired at all (e.g., see Editors, 1994). As an alternative, an alphanumeric format of data presentation or documentation (e.g., see Fuller et al. 1992; Acton et al., 1994) may be entirely adequate, if not preferable to graphical reporting formats. An example of such alphanumeric reporting format is given in the legend to Figure 9. As a matter of practice, such alphanumeric data, which may be obtained directly from the NCI-supplied mean-graphs, can be reported most appropriately in the "Experimental" or "Supplementary Material" sections in research publications. These data are in a form both convenient and fully adequate for future reference and analysis by any investigator. Moreover, such data need be published or otherwise documented only for the selected prototype or benchmark lead compound for a new series. Subsequent analyses, comparisons or related publications concerning new structurally or mechanistically related compounds, then need only refer to the published benchmark compound's screening fingerprint, along with any suitable qualifiers (e.g., COMPARE correlation coefficients).

Some other examples and suggestions for data reporting and interpretation, particularly as in this instance for a subpanel-selective lead and related compounds, are incorporated in Table 3. All compounds of the given series were tested in quadruplicate in several different concentration ranges. A preliminary examination of the resulting primary screening data revealed very few, if any, default values for any of the TGI concentrations for any of the cell lines in any tests of any of the given compounds. Furthermore, preliminary GI_{50}, TGI and LC_{50} analyses indicated that, in this instance, the most robust and reproducible correlation coefficients could be obtained with the TGI-based comparisons. Therefore, since the TGI mean-graphs sufficed both for potency as well as profile comparisons, Table 3 was constructed as shown. The average overall panel TGI concentration to a given compound was expressed as 1A] for each compound, whereas the selected subpanel-averaged TGI (for the brain tumor subpanel in this instance) for each compound was expressed as 1B]. A ratio of 1A]/1B] calculated for each compound provided a measure of subpanel selectivity independent of the accompanying COMPARE analysis. Gratifyingly, this measure proved consistent with the correlation coefficients comparing the members of the series to the selected benchmark compound. It is also noteworthy that, in this example,

the criteria for "selectivity", or lack thereof, were simply and arbitrarily defined by the investigators for this particular study, based upon the range of values obtained for the ratios and correlation coefficients (e.g., see footnotes to Table 3). It is reasonable that, in accord with the purpose of any given study, the responsible investigators may likewise define and apply their own criteria for selectivity based upon the particular data in hand for the series of compounds of interest. More complex, mathematically derived and/or statistically based criteria of selectivity may or may not add anything of further value, particularly if considered within the limits of the interpretability and practical utility of the NCI in vitro screening data.

Thus, Figure 9 illustrates the brain tumor subpanel specificity, and Table 3 reveals some structural requirements for such specificity of certain of the ellipticinium derivatives (Figure 8) tested in the NCI screen. Interestingly, none of the parent ellipticines (Figure 8) showed any subpanel specificity, although they were quite generally cytotoxic to all of the panel cell lines (Table 3). In contrast, a number of the corresponding, N-methyl quaternized ellipticiniums (Figure 8) showed selectivity comparable to the selected benchmark compound. These included compounds substituted at the 9-position with methyl, methoxy, and chloro, as well as the 9-unsubstituted compound. In sharp contrast, the 9-hydroxy compound was devoid of the selectivity (Table 3). The unusual in vitro brain tumor specificity of this ellipticinium lead was entirely unanticipated from any previously known biological activity of ellipticinium salts or parent ellipticines. Recent mechanistic investigations (Vistica et al., 1994; Kenney et al., 1994) of the prototype, 2-methyl-9-methoxyellipticinium acetate, indicate that its selective cytotoxicity to brain tumor cell lines is mediated, at least in part, by its preferential transport and accumulation in the sensitive cell lines. Other selective ellipticiniums also appear to be substrates for the transporter, but neither the nonselective 2-methyl-9-hydroxyellipticinium nor any of the parent ellipticines appear to be similarly transported. Thus, evidence to date is consistent with the view that certain ellipticinium derivatives identified by the NCI screen have a heretofore unknown in vitro tumor selectivity profile, and mechanism of action thereof.

FOR THE FUTURE

The data analysis methodologies and strategies emphasized herein have relied upon the generation of actual screening data on real compounds. A new emerging application of the screen is based upon the so-called "molecular characterization" of the cell line panel. In essence, this means the quantitative determination of differential expression in the panel cell lines of potential cell growth regulatory and/or drug sensitivity or resistance determinants such as oncogene or tumor suppressor gene products, growth factor receptors, transporters, and the like. This information is used to construct from a given set of molecular target data a hypothetical mean graph profile, in which all of the individual cell line deltas are scaled as appropriate in either direct or inverse proportion to the respective differential expression of the given molecular target in each cell line. Then, real compounds may be sought, using pattern-matching algorithms such as COMPARE, which have actual in vitro screening profiles matching the hypothetical one contrived upon molecular target data.

Some initial applications of this strategy have yielded promising results. For example, a hypothetical mean-graph fingerprint constructed from quantitative expression values for the *mdr*-1/P-glycoprotein in each of the panel cell lines was used as the seed to probe the NCI database (Alvarez et al., 1994). The COMPARE analyses yielded a series of compounds with screening profiles highly correlated with the constructed probe. Subsequent biochemical analyses confirmed that the selected compounds were indeed substrates for the P-glycoprotein. In a related study (Lee et al., 1994), similarly high correlations were found for the same compounds with respect to a probe constructed of rhodamine efflux values, which are functional assay counterparts of *mdr*-1 expression.

Some preliminary efforts have also been initiated to attempt to use the in vitro cell culture screening data as a means to discover heretofore unrecognized molecular targets (e.g., see Weinstein et al., 1994; Buolamwini et al., 1994). These types of studies may be of particular interest with respect to compounds already identified, and yet to be identified, which give novel fingerprints of differential in vitro growth inhibition or cytotoxicity in the NCI screen.

The discovery of new molecules acting at known molecular targets, and the discovery of new molecular targets modulated by new or known molecules, are complementary research goals, the pursuit of which may be effectively facilitated by practical applications of the NCI screen such as described herein. The ongoing development of other related and innovative approaches to data analysis (e.g., see Weinstein et al., 1992; 1994) will undoubtedly provide additional applications of general interest and utility.

Summary and Perspective

In this review we have focused upon a series of practical considerations and applications, at least some of which may be of interest to general users of the NCI screen whom we intended as our principal target audience. We have emphasized the potential utility of the NCI screen as an in vitro research tool to facilitate the discovery of novel new anticancer drug development leads. We have also attempted to illustrate the usefulness of the screen in SAR studies and lead-optimization research aimed both at achieving optimal potency while retaining a desired mechanism of action or unique spectrum of in vitro antitumor activity.

Throughout, we have intentionally avoided characterizing compounds as "active" or "inactive" *per se*, or of suggesting any specific definitions thereof. This is to further emphasize the use of the NCI screen as a research tool, ideally to be employed in complement to diverse other screening, drug discovery and research strategies, rather than proposing any more absolute (and perhaps meaningless) activity criteria. Instead, here we have adopted the perspective that "beauty is in the eyes of the beholder". More specifically, this is simply to suggest that investigators should use the NCI screening data, in concert with any other relevant available data or information from any source, in ways that are most meaningful or useful within the context of their own particular research projects and programs. Moreover, in such an individualized context it is not at all inappropriate that, subjectively or otherwise, the investigator may wish to define or adopt specific criteria for terms such as "active", "inactive", "selective", or "nonselective", for purposes of a given study that contains a decision point(s) dependent upon the assigned definitions. It is nonetheless always important that the individual investigator specify precisely what activity criteria definitions have been assigned or adopted in the particular study.

We have also offered some comments, suggestions and examples pertaining to establishing consistent standards for literature reporting or other documentation of NCI screening data. When desired or required *in lieu* of a graphical format, an alphanumeric reporting format is suggested particularly as an efficient and useful means of appropriately documenting the differential cytotoxicity fingerprints of a novel lead or prototype of a new mechanistic class. Some observations and cautions have also been provided concerning the methods by which NCI screening data are processed and incorporated into the standard screening data report; there are some potential pitfalls, of which the end-user should be aware and take into account in interpretations.

Finally, we wish to emphasize the placement of the NCI in vitro screen within the overall perspective of the anticancer drug discovery and development process. Ultimately, the NCI screen, or any screen for that matter, is useful only to the extent that it helps guide the discovery of useful new compounds with in vivo antitumor activity. To that end, the NCI in vitro screen also provides a practical means for the selection of compounds of interest for in vivo testing. The scope of the present review was intentionally limited to the NCI in vitro screen. However a preliminary progress overview (Grever et al., 1994) of the in vivo counterpart to the NCI in vitro screen suggests that the in vitro screen is an effective selector of compounds with in vivo anticancer activity. To what extent these in vivo active leads can be judged to be unique and important discoveries of the in vitro primary screen remains to be seen.

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